Hepatocellular carcinoma

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TITLE

Hepatocellular carcinoma: exploring the impact of ethnicity on molecular biology.

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ABSTRACT (150-250 WORDS)

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the third leading cause of cancer-related death. The high rate of diagnosis in non-curable stages and the lack of novel active treatments make it necessary to review all the possible sources of confounding results in this scenario. The incidence of HCC shows clear geographical variation with higher annual incidence in Asia and Africa than in Western countries; we aimed to review the literature to find if there are different trends in the main activated molecular pathways. Hyperactivation of RAS/RAF/MEK/ERK and PI3K/AKT/mTOR signalling and epithelial to mesenchymal transition (EMT) process are more prevalent in the Western population; however, fibroblast growth factor (FGF), transforming growth factor β (TGF β) and Notch pathways seems to be more relevant in Asian population. Whether these variations just reflect the distinct distribution of known causes of HCC or proper ethnical differences remains to be elucidated. Nevertheless, these clearly different patterns are relevant to regional or worldwide clinical trial design. If this information is neglected by sponsors and researchers the rate of failure in HCC trials will not improve.

KEYWORDS (MAX 6): hepatocellular carcinoma, molecular biology, differences, countries, response to treatment, sorafenib

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1.- Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the third leading cause of cancer-related death (1). Its poor prognosis is mainly related to high rate of diagnosis in non-curable stages, in which patients are suitable for palliative treatment. Sorafenib is the only treatment option available that will improve overall survival in patients with advanced HCC (2). A better understanding of the altered ??? biochemical pathways involved in HCC, clarifying the mechanisms by which the tumour evades treatment, seems to be the main way forward to improve clinical outcomes (3). Hepatocarcinogenesis is a complex multistep process, known to be mainly driven by chronic hepatitis which creates a pro-tumourigenic hepatic environment and leads to pro-oncogenic, epigenetic and genetic changes (4). The molecular biology of carcinogenesis and tumour progression of HCC has been increasingly understood during the last decades. Several important intracellular pathways, such as the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways; growth factors such as fibroblast growth factor (FGF); and critical processes such as epithelial to mesenchymal transition (EMT) and angiogenesis seem to be involved in the development of HCC (5, 6). Indeed, an important hallmark of HCC seems to be the absence of a clearly identified addiction to an oncogene or pathway.

The blockage (? inhibition) of some of these pro-oncogenic pathways has led to targeted therapy options, such as sorafenib (7, 8). However, the clinical benefit to systemic treatment in HCC seems to differ between countries (8). In addition, the incidence of HCC shows clear geographical variation with higher annual incidence in Asia and Africa than in Western countries (9). When analysing the different next generation sequencing projects, it is noticeable that there are at least different patterns of gene-alterations depending on the different areas of the world (10-13). As an example, we plot the percentage of mutations per sample for the 100 most altered genes extracted from the International Cancer Genome Consortium (ICGC) data portal (14), see **Figure 1**. One of the postulated explanations for these facts could be (? pre-existing) molecular discrepancies throughout different ethnicities.

The aim of this review is to explore the evidence supporting the potential biological differences across the world. Do they really exist? If they do, how relevant are they and how much evidence do we have? What are the implications for trial design?

2.-Molecular pathways

A. RAS/RAF/MEK/ERK pathway

This pathway is one of the key signalling cascades involved in the development of multiple cancers, resulting in cell cycle progression, apoptosis resistance, extracellular matrix remodelling, cellular migration and angiogenesis. As previously described in other malignancies, *EGFR* (epidermal growth factor receptor), K-RAS (Kirsten rats sarcoma) and B-RAF (rapidly accelerated fibrosarcoma homolog B) mutations are the main mechanisms for the activation of this pathway. Analysis of *RAS* (rats sarcoma) gene mutation has been widely explored in multiple ethnicities in HCC. *RAS* mutations did not seem to play an important role in HCC carcinogenesis in African Blacks (15). Even though the mutations of *RAS* family genes are infrequent in Asian population (1.7-5.6% *K-RAS*, 0% *B-RAF*, 0-71% *H-RAS* (Harvey rats sarcoma), 0% *N-RAS* (rats sarcoma neuroblastoma homolog)) (16-19), mutation rate seems to be higher in Western population (2-16% *K-RAS*, 0-23% *B-RAF*, 14% *N-RAS*) (20-23). One of the highest rate of *K-RAS* mutation (42%) has been described in Western workers exposed to vinyl chloride, which seems to have a direct toxic effect in the hepatocytes (24). It is worth mentioning that there seems to be a significant variation in the mutation rate not only between ethnicities, but also between studies within the same population group (i.e. range for *H-RAS* mutation varies from 0 to 71% in Asian population (20-23)), which is probably related to study sample size and sequencing technique employed. Finally, and based on the higher rate of *EGFR* mutation rate found in non-small cell lung cancer in Asian population, the status of *EGFR* has also been explored. Up-to-date, no mutations in *EGFR* have been described in Asian population (25); no information is available in non-Asian population.

Looking for an explanation for the benefit from sorafenib (inhibitor of RAS-RAF-MEK-ERK pathway) and the low rate of *EGFR/RAS* mutations in HCC; Calvisi *et al* explored this pathway's downstream status in *K-RAS* wild type HCC patients. The authors found that in the absence of *K-RAS* mutations, downregulation of at least one RAS-GTPase activating proteins exist, in keeping with alternative mechanism of hyperactivation of this pathway in HCC (26).

B. PI3K/AKT/mTOR pathway

The phosphatidylinositol 3-kinase (PI3K) cascade is a critical component of survival signalling. PI3K-activated AKT (AK thymoma) (phosphorylated AKT) inhibits cell death pathways by inactivating pro-apoptotic proteins. mTOR (mammalian target of rapamycin) pathway is known to be activated in viral hepatitis and HCC (27). Multiple Asian and Western studies have analysed the status of this pathway in HCC.

Low number of mutations within the mTOR pathway have been described in Asian population (0% *PTEN* (phosphatase and tensin homolog), 0-1.5% *PI3CA* (phosphatidylinositol 3-kinase catalytic subunit α), (16, 18)). Chen *et al* analysed 200 HCC samples for the mTOR pathway status employing immunohistochemistry: PTEN loss was correlated with phospho-AKT and phospho-mTOR corresponding to pathway activation (28). Moreover, PTEN expression was more frequently lost in HCC (around 48-63% of HCC samples were positive for PTEN), when compared with surrounding normal liver (89%) (29-32).

According to the published Western studies, the rate of mutations in mTOR pathway seems higher in this population (7-50% *mTOR*, 7-28% *PI3CA*) (20, 22, 33). This activation in the mTOR pathway (present in around 15-40% of HCC) leads to an increasing angiogenesis and progression in HCC (34-36) and is a plausible opportunity for enrichment clinical trials with PI3K/AKT/mTOR pathway inhibitors.

C. HGF/MET pathway

C-MET (mesenchymal epithelial transition factor gene) is a proto-oncogene that encodes the hepatocyte growth factor receptor (HGFR or MET): a regulator of the invasive growth of tumours. MET is normally expressed by cells of epithelial origin, while expression of HGF (ligand of MET) is restricted to mesenchymal cells. MET activation by HGF binding induces MET kinase catalytic activity, and consequently promoting tumour growth, angiogenesis and metastasis with an activation of the EMT.

Osada *et al* showed that the presence of higher serum levels of HGF and higher tumoural MET expression were related with more aggressive HCC (37). The most accurate study analysing the MET expression in HCC is the phase II trial of tivantinib (oral MET inhibitor), published in 2013, where 77 patients were included (89% white, 5% Asian) (38). In this phase II study, all patients had analysed the MET status at study entry, which was reported as follows: Western (51.4%) and Asian (66%). This phase II trials concluded that MET expression was a poor prognostic factor in HCC patients, and further efficacy trials are ongoing with this target. A further clinical trial with Tivantinib is ongoing in Asian population and will be able to give more accurate information about these potential differences (NCT02029157).

D. FGF (fibroblast growth factor) signalling

The FGF family of proteins, consisting of four tyrosine kinase receptors (FGFR) and more than 20 ligands, is involved in several processes of malignant transformation of the liver (39). FGF19 in particular, ligand of FGFR4, has emerged as an oncogene from an oncogenomic screen performed in mice by Sawey *et al* (40). Recent publications pointed out that *FGF19* amplification occurs in later stages of liver cancer. Events affecting *FGF19* gene in the Western population occurs in about 5-14 % of the cases and co-amplification with *CCND1* (cyclin D1) seems to confer a worst prognosis (40). On the other hand, a protein expression analysis of FGF19 in an Asiatic cohort showed an expression rate of about 45% and it was associated with early recurrence and shorter survival (41). The reviewed data did not clarify the impact of ethnical or regional differences in this particular pathway.

E. IGF (insulin growth factor) family

IGFR (insulin-like growth factor receptor) signalling is activated in 20% of patients with HCC through several mechanisms, including overexpression of the oncogenic ligand IGF2, and deregulation of the IGF binding proteins (IGFBP) IGFBP2 and IGFBP3 (42). A relationship between cirrhosis and IGF pathway activation has been described (43, 44), together with a correlation with worse prognosis and shorter time to progression (45, 46). IGF pathway activation seems to be present in both populations: expression of IGFR2 has been described in 34% and 100% of Asian (47) and Western (48) HCC patients, respectively. Moreover, 21% of patients with Western HCC showed features in keeping with pathway activation (42). Nevertheless, the attempts to target this pathway clinically failed due to futility (49).

F. JAK/STAT pathway

Mutations in *JAK* (Janus kinases) 1 are found in about 1% of the HCCs. This pathway seems to be related to chronic inflammation that could be triggered by pro-inflammatory cytokines such as IL-6 (interleukin-6). This has been shown elegantly by zebrafish model harbouring human IL-6 published by Jung *et al* (50). A constitutive activation of JAK/STAT (signal transducer and activator of transcription) pathway dependent on aberrant hypermethylation of *SSI-1* (stress survival islet-1) gene has been identified in a significant percentage of HCC patients, both in Western (65% (51)) and in Asian populations (53% (52)). Interestingly, restoration of the pathway

by administration of JAK2 inhibitors was able to induce apoptosis and could be an interesting target for future options of treatment (53).

G. TGF β signaling

Transforming growth factor β (TGF β) is a family of growth factors with a pleiotropic presence ? influence involved in multiple physiological processes ranging from morphogenesis to immunosuppression. It has a dual effect in HCC, suppressing carcinogenesis and cell growth at early stages, whilst potentiating EMT, angiogenesis, tumour progression, invasion and metastasis in advance tumour stages. When measured in peripheral blood, levels of TGF β vary significantly within the available literature (54), which may be expected due to the heterogeneity of patients ? assessed. Interestingly, when comparing three studies with similar populations, the Western study showed a maximum level of TGF β of 3.7 ng/mL (55), which was significantly lower compared to the levels identified in two Asian studies (40 ng/mL and 65 ng/mL, respectively (56, 57)). Acknowledging the difficulty of comparing retrospective studies with different methodologies, it can be argued that ethnicity may explain some degree of the differences identified, as postulated previously by Lin *et al* (54).

H. P53 tumour suppressor signalling

Aberrations in *TP53* (tumour protein 53) gene, known as one of the main aberrations involved in cancer development, seem to be more frequent in HCC population with high exposure to aflatoxin (58). *TP53* mutations have been identified in 33-37% and 15-16% of HCC patients in Asian (59, 60) and Western population (61-63), respectively. In both patients groups, *TP53* mutations seems to be related with worse survival (60-63).

I. Telomere maintenance

Telomere abnormalities appear to play a role in tumour promotion and maintenance. These molecular aberrations have been described in around 44-85% of HCC samples across Asian (64-66) and Western (67) populations. While the rate of telomere abnormalities is not influenced by the cause of HCC (hepatitis B virus (HBV), hepatitis C virus (HCV) or alcohol), the underlying molecular mechanism seems to be cause-specific: this has been shown in a series of 40 Western patients (67) matched with the results in a population of 58 Asian HCC (65). In both populations of patients, the presence of telomere abnormalities is associated with worse prognosis.

J. AAV2 virus integration into the genome

Lately, clonal integration of the adeno-associated virus type 2 (AAV2) has been reported in 11 cases of 193 Western HCCs (68). Generally, integration was found in known cancer driver genes such as, *CYCLIN A2*, *telomerase reverse transcriptase* and *CYCLIN E1*, amongst others. There is no data from Asian cohorts of patients in order to discuss the ethnic variability of this particular finding.

K. EMT: Epithelial to mesenchymal transition

The WNT/ β CATENIN pathway is involved in the regulation of cell invasion and migration. Nuclear expression of β CATENIN has been shown to impact on prognosis in other malignancies. The expression of WNT/ β CATENIN is better described in Western than in Asian population: 42% and 7% of HCC have shown mutations in *WNT* (wingless-type MMTV integration site family member) and *CTNNB1* (gene coding for β -CATENIN) respectively in some of the Western series (20, 62). In the Asian population, a tumour stroma gene-signature was able to predict clinical outcome based on 122 patients in whom the stroma biology was analysed (69).

L. Angiogenesis

In 2009 Schoenleber and colleagues published a systematic review and meta-analysis of the available studies analysing the prognostic impact of VEGF (vascular endothelial growth factor) (70) both in serum or tumoural tissue in HCC population (71). High tissue VEGF levels predicted poor overall (HR (Hazard Ratio) 2.15, 95% CI (Confidence Interval): 1.26–3.68) and disease-free (HR 1.69, 95% CI: 1.23–2.33) survival. Similarly, high serum VEGF levels predicted poor overall (HR 2.35, 95% CI: 1.80–3.07) and disease-free (HR 2.36, 95% CI 1.76–3.16) survival. Fifteen out of 16 studies included were undertaken in Asian populations, while only one was undertaken in Western patients (72). The meta-analysis concluded that the differences regarding the origin of the population of the trials was not entirely unexpected, given the high burden of HCC in Asian populations. However, a query regarding external validity and applicability of the results to Western populations was raised. In 2013, Yegin *et al* published a 78 patient HCC series showing that serum VEGF level was an independent predictor of survival in advanced HCC in a Turkish population (73); while other Asian studies have confirmed its impact in predicting response to trans-arterial chemoembolization in Asian population (70, 74).

M. Chromatin Remodelling (ARIDs)

ARID2 is a subunit of the polybromo and BRG1 associated factor (PBAF) chromatin remodelling complex which facilitates ligand dependant transcriptional activation by nuclear receptors (75). The physiological role of these complexes is to modify the chromatin structure and therefore indirectly alter the transcription of the genes involved. Li *et al* reported the mutation rate of *ARID2* and *ARID1A* in Asian HCC patients to be around 3-18% and 4-17%, respectively (76). *ARID2* mutations showed higher penetrance within HCV-associated than HBV-induced HCC (76), however ethnicity could not be completely ruled out as a factor impacting on these differences. Mutations in *ARID2* were correlated with *CTNNB1* (gene coding for β -CATENIN) mutations but mutually exclusive with *TP53* mutations. Mutations in the histone methylation writer family (*MLL*) are also found in a range of 2-10% both in Asian (77) and Western populations (78, 79). There are no clear differences in the regional incidence of these alterations that could be more likely matching the distribution the underlining pathological cause of the cancer.

N. Stemness properties

NOTCH (Neurogenic locus notch homolog) pathway has been widely studied in Asian population, while no publication is available for Western patients. The poor prognosis of NOTCH pathway activation is a reproduced result across most of the available Asian series (80-82). One of the largest cohorts included 288 HCC patients and shows that the cytoplasmic expression of NOTCH1, cytoplasmic expression of NOTCH3, coexistent nuclear expression of NOTCH3, and cytoplasmic NOTCH4 overexpression determined by immunohistochemistry were observed in 145 (50.3%), 60 (20.8%), 17 (5.9%), and 172 (59.7%) of the samples respectively (82). In this series, NOTCH1 and NOTCH 4 had an impact in poorer survival, encouraging the use of NOTCH for predicting survival. NOTCH activation has also been related to a higher rate (? incidence) of distant metastases, which is postulated to be explained by activation of NOTCH/SNAIL1/E-CADHERIN pathway (83, 84).

In addition, Kuo *et al* published results from a series with 80 patients diagnosed with HCC where the expression of HEYL (hairy/enhancer-of-split related with YRPW motif-like) protein was analysed as a downstream molecule of the NOTCH and TGF β pathways (85). Authors showed that *HEYL* gene was inactivated in more than 75% of HCC samples with a significant reduction in apoptosis.

O. Hormone receptors

The expression of hormone receptors has been investigated widely in both populations. Wang *et al* analysed the expression of estrogen (ER) and androgen (AR) receptors in 32 Asian HCC patients using RT-PCR (reverse transcription polymerase chain reaction) (86). Wild-type ER- α and AR were expressed in all of the samples investigated. Expression of the ER- α variant was independent of gender and HCV and HBV status. In contrast, wild-type ER- β was expressed more often in HCV patients than in HBV patients (95.7% vs. 44.4%; $p < 0.05$). Within Western population, 33-46% of HCC samples showed overexpression of ER (87-89). In summary, aberrant ER, both in Asian (90) and Western (91) population have been described and shown to have an impact in patients prognosis.

P. Somatostatin receptors (SSTs)

HCC has shown to express SSTs both in Asian (92, 93) and Western (94, 95) population in around 50% of HCC patients when analysed with immunohistochemistry. The rate of octreotide scan positivity has not been studied in Asian population, while in the Western population it seems to be between 35-56% (96, 97), matching with the rate of positivity in the immunohistochemistry studies.

3.- Discussion of findings and implications for trial design

As specified, hyperactivation of RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways and the presence of EMT are more prevalent in the Western population; FGF, TGF β and NOTCH pathways seem to be more relevant in Asian population. The role of angiogenesis, expression of somatostatin and hormonal receptors, aberrations in HGF/MET, telomere maintenance and WNT/ β CATENIN seem to be similar in both groups of patients. See graphical representation summarising our finding in **Figure 2**.

Hepatocellular carcinoma remains challenging for clinicians and researches who try to improve its management and outcome. Unfortunately, the failure of the STORM trial in the adjuvant setting is a clear example of this statement (98). HCC is most probably one of the cancer types where the dynamic relation between the transformed cells and the stroma during the evolution of the disease is more patent and remains cryptic when talking about treatment targets. We would like to provide the ? reader of this review with a tool to ? clarify the areas to focus on when planning

a clinical trial depending on the target population by ethnicity and when planning a worldwide trial with an appropriate stratification.

Multiple ongoing clinical trials are currently trying to improve our management of HCC patients (99). Four-hundred and sixty-five trials are currently recruiting patients all around the world; from those 465 trials, 179 are employing systemic treatments (See **Table 1** and **Figure 3**). The main countries with ongoing clinical trials are East Asia (228 ongoing studies), followed by Europe (101 ongoing studies) and United States (124 ongoing studies) which shows a balanced recruitment between these two main populations. According to the current numbers, the rate of trials employing systemic treatment seems to be higher in Europe (84.1%) and North America (46.7%) compared with East Asia (32.3%). No clinical trials are planned for direct comparison of outcome between Asian and Western population but the results of some of the largest randomized phase III trials may be able to clarify our interpretation of ethnicity impact in response to treatment.

In the era of precision medicine, realistic and achievable trials are key to the applicability of molecular biology discoveries. Small Phase II trials with enriched population could give clinicians the first signs of such activity. There is a need to be conscious of the sub-population we are targeting when planning these studies. For instance, even in the presence of thorough rationale, trying to develop a trial targeting a specific molecular aberration in a population of patients with low prevalence of that specific target could mean failure by slow recruitment and early trial closure. The findings summarised in this review could inform future clinical trial designs, facilitating the selection of the most prevalent molecular aberration to target according to the population of interest.

4.- Conclusion

In summary, the research available in HCC is geographical heterogeneous. There is no available study comparing ? ethnic populations, and definitive conclusions cannot be made from the direct comparisons of the results detailed above. Specific pathway however seems to be more activated in certain populations and this should be taken into account during trial design and feasibility assessment.

5.- Figures and Tables

Figure 1: Differential analysis of mean percentage of mutated gene per sample (100 genes with the highest rate of mutation have been selected for this graphical representation; symbol ? axis lists contents name of only 20 genes for visualisation purposes; please refer to Supplementary Material 1 for full list of genes and percentage of altered genes represented in this graphic). Figure 1 shows differences between Asian (blue) and Western (red) data. Data from International Cancer Genome Consortium were employed in this graphic; specifically, data from LICA-FR (10), LIHC-US (11), LINC-JP (12) and LIRI-JP (13) studies are shown.

Figure 2: Summary of findings regarding molecular differences between Asian (blue) and Western (red) hepatocellular carcinoma patients. The size of the font is proportional to the relevance or prevalence of each pathway in each population.

Region	Current clinical trials in HCC	Studies focused on systemic treatment of HCC	Other studies
World	465	179	286
Asia			
East Asia	228	74	154
China	119	34	85
Japan	20	14	6
South Asia	8	2	6
Southeast Asia	19	11	8
Pacifica	11	6	5
Western			
Europe	101	85	16
Middle East	13	3	10
Russia	5	4	2
Canada	23	8	15
Mexico	6	2	4
United States	124	58	66
South America	9	4	5
Africa	3	2	1
Egypt	2	2	0

Table 1: Ongoing clinical trials in hepatocellular carcinoma (99).

Figure 3: Geographical distribution of ongoing clinical research in hepatocellular carcinoma. The map shows the number of clinical trials opened in each country: both trials with systemic and local therapies are included. Adapted from (99).

Supplementary material 1:

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